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EXPERIMENTAL INFECTION OF SHEEP WITH *NEOSPORA CANINUM* OOCYSTS

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ABSTRACT: The purpose of the present study was to investigate the potential of *Neospora caninum* oocysts to infect sheep and determine whether *N. caninum* DNA could be detected by polymerase chain reaction (PCR) assay in blood and brain of sheep after oocyst inoculation. Six ewes were inoculated per os with 10^4 *N. caninum* oocysts, whereas 2 ewes served as uninoculated controls. All sheep were bled weekly for 7 wk after inoculation. Blood was analyzed for the presence of *N. caninum* DNA by 2 different PCR assays, as well as for the presence of antibodies to recombinant and native *N. caninum* antigens. *Neospora caninum* DNA was detected in 2 sheep as early as 7 days after oocyst inoculation (DAOI). All 6 sheep were PCR positive by 32 days and remained positive until the end of the study at 49 DAOI. Aside from 1 ewe, all sheep inoculated with *N. caninum* oocysts contained detectable *N. caninum* DNA in the brain tissue collected at 49 DAOI. Unlike with PCR, no lesion or parasite was detected by immunohistochemistry. Antibodies were detected by enzyme-linked immunosorbent assay, *Neospora* agglutination test, or immunoblotting to either native or recombinant *N. caninum* antigens in sheep inoculated with oocysts.

Neospora caninum is considered to be an important cause of reproductive failure in cattle worldwide (for review, see Dubey, 1999). Infection of the fetus has many possible outcomes from fetal resorption to birth of infected but otherwise healthy calves. Abortion is a direct result of congenital transfer of *N. caninum* tachyzoites to the fetus during gestation (Björkman et al., 1996; Anderson et al., 1997). These tachyzoites may have arisen from a reactivated latent *N. caninum* infection or by ingestion of *N. caninum* oocysts. Because of the high costs and long gestation time associated with cattle, a number of researchers have investigated the use of sheep as a ruminant model for bovine neosporosis. Natural or experimental infection of sheep with *N. caninum* tachyzoites leads to clinical and pathological signs similar to those observed in cattle (Dubey and Lindsay, 1990; Dubey et al., 1990; McAllister et al., 1996; Buxton et al., 1997; Buxton, 1998; Buxton et al., 1998; Jolley et al., 1999; Kobayashi et al., 2001; Koyama et al., 2001). Similar to cattle (Innes et al., 2000, 2001), there is evidence for the development of protective immunity against *N. caninum* infection in sheep (Buxton et al., 2001). Although transplacental transmission is a known route of infection, little research has been conducted on infection of ruminants with *N. caninum* oocysts (De Marez et al., 1999). The purpose of the present study was to examine the early infection dynamics and immune response of sheep to experimental *N. caninum* oocyst inoculation.

MATERIALS AND METHODS

Neospora caninum oocysts of the NC-2 isolate were obtained from the feces of a dog as described by De Marez et al. (1999) and were inoculated per os into sheep 2 mo after collection and purification. The infectivity of the inoculum was verified by bioassay in gerbils (Dubey and Lindsay, 2000). A 1:10 dilution of the inoculum given to sheep was infective for gerbils.

Six 3- to 4-mo-old ewes (nos. 3141, 3144, 3148, 3151, 3155, 3156) were inoculated per os with approximately 10^4 *N. caninum* oocysts; 2 ewes of the same age (nos. 3143, 3146) served as uninoculated controls. Neither infected nor control ewes had demonstrable antibodies to *N. caninum* before the start of the study. The sheep also showed no titer to *Toxoplasma gondii* in 1:25 dilution of their sera in the modified agglutination test (Dubey and Desmonts, 1987). The sheep were bled before infection and weekly for 7 wk after oocyst inoculation at which time they were killed using a stun gun. Necropsy examination was done on all sheep, and brain, spinal cord, lungs, heart, liver, spleen, skeletal muscle, tongue, and kidney were collected.

A portion of each tissue was fixed in 10% buffered neutral formalin and embedded in paraffin from which 5-μm tissue sections were prepared. Sections were subjected to hematoxylin and eosin staining or deparaffinized and reacted with anti–*N. caninum* antibodies using procedures described by Lindsay and Dubey (1989).

As a means of determining levels of *N. caninum* present in various tissues, DNA was extracted from a portion of tissue and subjected to polymerase chain reaction (PCR) directed to the Nc5 or 14-3-3 sequence using procedures described by Liddell et al. (1999a). Amplification reactions were subjected to polyacrylamide gel electrophoresis, ethidium bromide staining, and image capture to a CCD camera. An internal standard was included in each amplification reaction to control for false-negative PCR (Liddell et al., 1999b). Appropriate positive (*N. caninum* tachyzoite DNA) and negative (H_2O) controls were included in each set of reactions.

Sera from all sheep were tested for antibodies to *N. caninum* using a recombinant NcGRA6/NcGRA7 antigen enzyme-linked immunosorbent assay (ELISA), as described by Jenkins et al. (1997). The recombinant proteins were applied to high-binding Immulon II microtiter plates at 50 ng per well. Sera were diluted 1:500 in PBS-Tw20 and applied in duplicate wells for 2 hr at room temperature (RT). After washing with PBS-Tw20, 100 μl peroxidase-labeled rabbit anti-sheep Ig (Sigma Chemical Co., St. Louis, Missouri) was applied at a 1:1,000 dilution to each well for 1 hr at RT. The level of antibodies to recombinant NcGRA6/NcGRA7 was measured by addition of 50 μl peroxidase substrate (0.1 mg/ml *o*-phenylenediamine–0.01% H_2O_2). Reactions were stopped by the addition of 50 μl of 1% H_2SO_4 and measured on a microtiter plate reader at 492 nm.

Neospora caninum tachyzoites (NC-1 strain, Dubey et al., 1988) were maintained in human foreskin fibroblast cells and harvested using procedures described by Liddell et al. (1999a). Sera were diluted 2-fold, starting at 1:25, and examined by *Neospora* agglutination test (NAT) as described by Romand et al. (1998). Paraformaldehyde-fixed *N. caninum* (NC-1) whole tachyzoites were used as antigen in the NAT.

Antisera from all sheep were also tested by immunoblotting to identify specific *N. caninum* antigens recognized after immunization or tachyzoite challenge. *Neospora caninum* tachyzoites were harvested as described by Liddell et al. (1999a), pelleted by centrifugation, and resuspended in phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors (Boehringer-Mannheim, Indianapolis, Indiana). A volume equivalent to 10^7 *N. caninum* tachyzoites was mixed with an equal volume of sample buffer (Laemmeli, 1970), electrophoresed by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to Immobilon membrane using a semidry blotter (BioRad, Richmond, California). The membranes were treated with PBS–2% nonfat dry milk for 1 hr at RT to block nonspecific antibody binding in subsequent steps. After blocking, the membranes were cut into individual strips and incubated for 2 hr with a 1:250 dilution of

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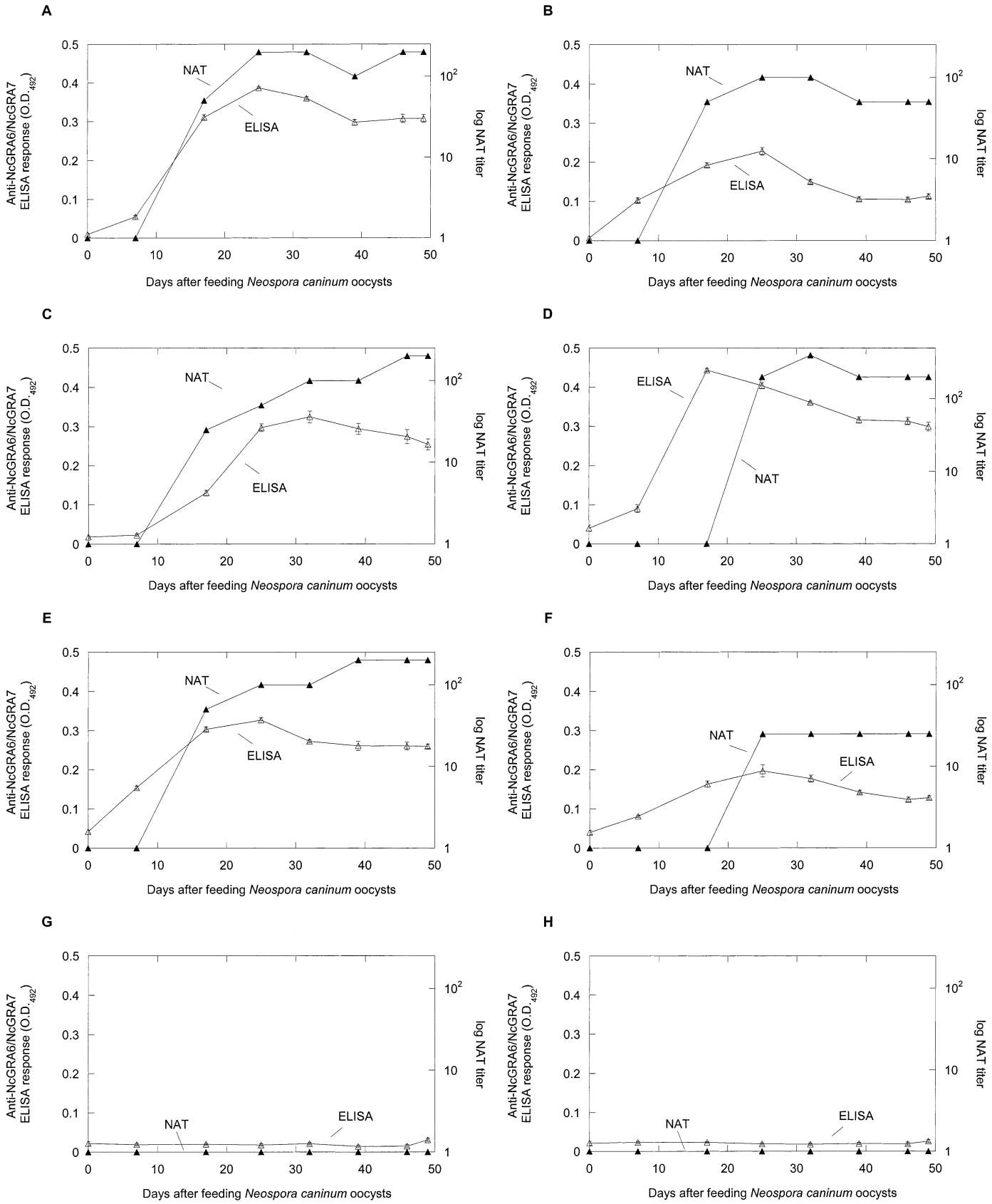

FIGURE 1. Antibody response over time to *Neospora caninum* antigen in ewes inoculated with *N. caninum* oocysts as measured by recombinant NcGRA6–NcGRA7 ELISA or NAT. Infected animals: **A,** ewe no. 3141; **B,** ewe no. 3151; **C,** ewe no. 3144; **D,** ewe no. 3155; **E,** ewe no. 3148; **F,** ewe no. 3156. Uninfected controls: **G,** ewe no. 3143; **H,** ewe no. 3146.

pooled sera from uninoculated control or from *N. caninum* oocyst–inoculated sheep. Binding of sheep antibody was assessed by treating membranes with 0.5 mg/ml biotinylated rabbit anti-sheep Ig (Sigma) followed by 0.25 mg/ml avidin peroxidase (Sigma). The immunoblots were developed by incubating with 0.5 mg/ml 4-chloro-1-napthol and 0.015% H_2O_2. The strips were washed 3 times between each step with PBS-Tw20.

RESULTS

PCR directed to 2 different *N. caninum*–specific DNA sequences detected *N. caninum* DNA in blood from 2 of 6 sheep by 25 days after oocyst inoculation (DAOI). The PCR was positive for all 6 sheep by 32 DAOI and remained positive until the end of the study at 7 wk postinoculation (p.i.). Aside from 1 infected ewe (no. 3141), all animals had detectable *N. caninum* DNA in brain tissue at 7 wk p.i. Neither blood nor brain tissue was positive for *N. caninum* in uninfected controls. Although PCR showed clear evidence for *N. caninum* infection, no lesion or parasite was seen in tissues of any of the 8 sheep by immunohistochemical staining.

Sheep inoculated with *N. caninum* oocysts also exhibited a rapid increase in anti-NcGRA6/NcGRA7 levels by 7 DAOI (Fig. 1A–F). The anti-NcGRA6/NcGRA7 levels peaked at about 25 DAOI and showed a gradual decrease over time during the remainder of the study (Fig. 1A–F). Uninfected control sheep remained negative throughout the study (Fig. 1G, H).

Although NAT titers did not increase as rapidly as the anti-NcGRA6/NcGRA7 levels, all sheep were NAT positive by 25 DAOI (Fig. 1A–F). Uninfected control sheep remained negative by NAT throughout the study (Fig. 1G, H).

By immunoblotting, sera from *N. caninum*–infected sheep recognized a 41-kDa tachyzoite protein as early as 7 DAOI (Fig. 2A). By 17 days, major protein bands at 56, 73, and 100 kDa as well as a number of minor proteins were identified by sera from *N. caninum*–fed ewes (Fig. 2A). Beyond 32 days, the response was directed to the 41-, 56-, 73-, 100-kDa as well as to a 28-kDa antigen (Fig. 2A). There were only slight differences in the recognition pattern of these antigens by individual ewes (data not shown).

DISCUSSION

The current study demonstrated that sheep inoculated with *N. caninum* oocysts contained detectable levels of *N. caninum* DNA in circulating blood as early as 7 DAOI. Similar to findings in cattle (De Marez et al., 1999), *N. caninum* DNA is detectable in brain tissue of sheep infected with *N. caninum* oocysts. The absence of detectable lesions or parasites in any tissue from infected sheep is consistent with results from studies in mice that showed PCR to be more sensitive than histochemical techniques (unpubl. obs.). This study also suggests that PCR detection of *N. caninum* in blood may be a sensitive method for diagnosing neosporosis.

Similar to *N. caninum* tachyzoite infection of sheep (Buxton et al., 1997, 1998), ewes inoculated with oocysts mounted an immune response to *N. caninum* tachyzoite antigen, as measured in rELISA, NAT, and immunoblotting. Quite interesting is the fact that the response to recombinant NcGRA6/NcGRA7 peaked early (25 days) after infection and decreased gradually during the rest of the study. In contrast, NAT titers increased gradually during the infection and peaked by 25–39 DAOI.

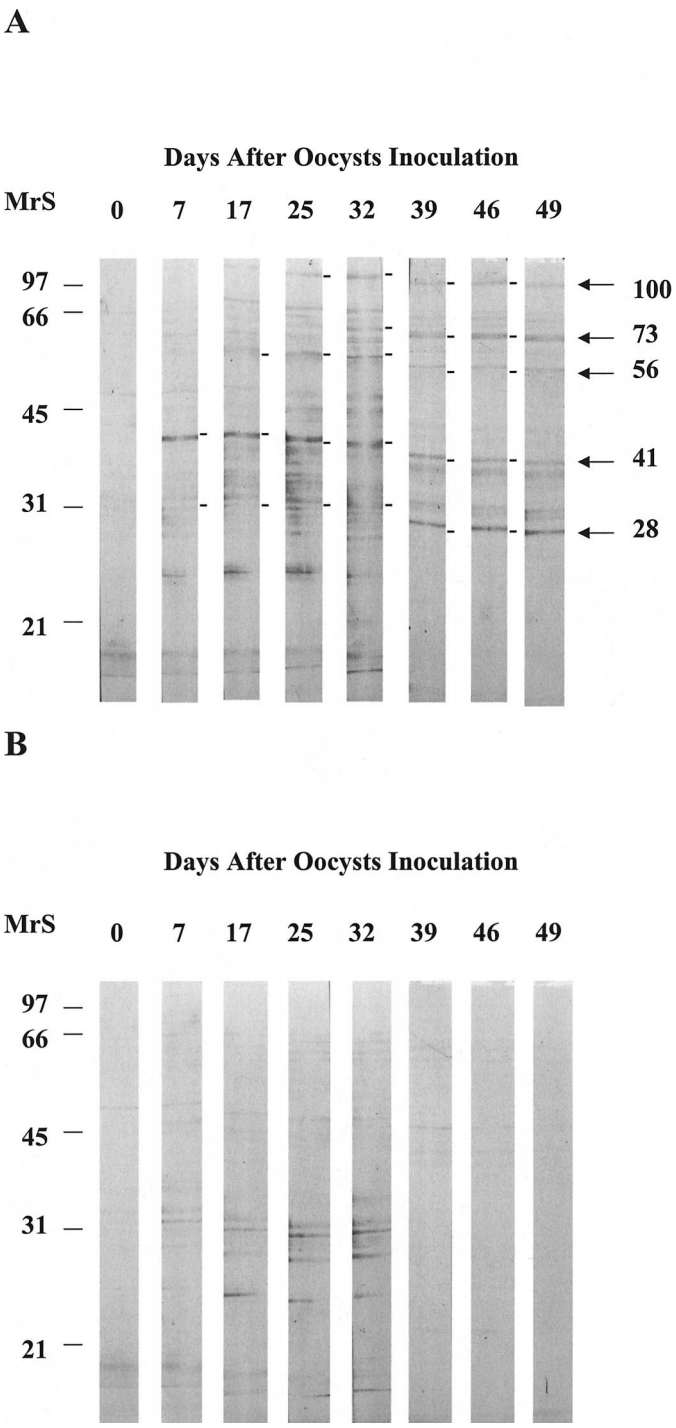

FIGURE 2. Antibody response over time to *Neospora caninum* antigen in ewes inoculated with *N. caninum* oocysts as measured by immunoblotting assay to *N. caninum* tachyzoite antigen. Immunoblotting results were obtained using pooled sera from infected (**A**) or uninfected (**B**) ewes. MrS, low-range molecular weight standards (BioRad). Please note that sera from 0 to 32 DAOI were probed on a different blot than sera from 39 to 49 DAOI.

Similar results were observed in cattle inoculated with *N. caninum* oocysts (De Marez et al., 1999). It is possible that antibodies to dense granule proteins are elicited early in infection, whereas antibodies to surface proteins (as measured by NAT) are elicited later. Because of the background binding by sera from uninfected sheep to *N. caninum* tachyzoite antigen in the native NcGRA6/NcGRA7 size range (33–37 kDa), it is difficult to ascertain differences in intensity over the infection period to the native NcGRA6 or NcGRA7 protein. It is interesting that a 41-kDa tachyzoite protein is recognized so early after oocyst inoculation and that the intensity of binding decreases with time. The nature of this protein and whether it is identical to the 40- or 41-kDa protein identified by others is unknown (Bjorkman and Hemphill, 1998; Schares et al., 1999; Sonda et al., 2000).

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